Lasers in Medical Science (2019) 34:1889–1895
https://doi.org/10.1007/s10103-019-02787-4

ORIGINAL ARTICLE

Effects of laser-assisted thinning versus opening on clinical outcomes according to maternal age in patients with repeated implantation failure

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Received: 12 January 2018 / Accepted: 12 April 2019 / Published online: 1 May 2019

Abstract
Laser-assisted thinning (LAT) and laser-assisted opening (LAO) are performed as part of human in vitro fertilization (IVF) to increase the implantation rate in patients with a poor prognosis and in cases of repeated implantation failure. However, an insufficient number of studies have directly compared LAT and LAO using the same methods. Therefore, we compared the effects of LAT and LAO on clinical outcomes according to maternal age in patients with repeated implantation failure. This retrospective study was performed in 509 IVF cycles (458 patients). The cycles were divided based on maternal age and the method used (< 38 years LAT, $n = 119$ vs. LAO, $n = 179$ and ≥ 38 years LAT, $n = 72$ vs. LAO, $n = 139$). Cleavage-stage embryos before transfer were either thinned or opened using a 1.46-μm noncontact diode laser. We compared the implantation rates and pregnancy outcomes of cycles between LAT and LAO according to maternal age. The characteristics of patients did not differ significantly among the groups ($p > 0.05$), with the exception of mixed factor infertility, which was more common in the LAT group than in the LAO group among patients < 38 years of age (10.1% vs. 2.8%, $p = 0.008$). The LAT and LAO groups showed similar rates of biochemical pregnancy, clinical pregnancy, ongoing pregnancy, abortion, implantation, singleton pregnancy, and twin pregnancy ($p > 0.05$). In conclusion, LAT and LAO had similar clinical outcomes. Therefore, we did not find any evidence that LAT is superior to LAO. In fact, the patients ≥ 38 years of age who underwent LAO tended to have a lower abortion rate. Further study is necessary to confirm these results in a larger population.

Keywords Laser-assisted hatching · Laser-assisted thinning · Laser-assisted opening · Zona pellucid · Pregnancy outcomes

Introduction

Assisted hatching (AH) is a technique used in assisted reproductive technologies to imitate the natural embryo-hatching procedure. AH has been used since the late 1980s to improve the low rates of implantation and clinical pregnancy in patients with a poor prognosis and embryos with a thick zona pellucida (ZP) [1]. Since then, several studies have reported the use of AH to treat embryos with chemical or physical ZP changes in patients with conditions such as advanced maternal age [2, 3] and repeated implantation failure (RIF) [4, 5].

AH involves artificial removal of the ZP, and a variety of AH methods have been applied, including zona thinning and zona drilling/opening (digging a hole) using chemical, mechanical, or laser energy [6]. Lasers are advantageous in comparison to chemical or mechanical opening procedures because they allow the target to be approached easily, with minimal absorption of energy by the embryo. In the laser-assisted hatching (LAH) process, the target can be accurately controlled, and the LAH has been shown to allow the creation of openings in the ZP without mechanical or mutagenic side effects [7, 8]. Although laser systems are among the most expensive pieces of equipment in the laboratory, they enable easier, faster, and safer micromanipulation of the ZP, with consistency among users [9]. LAH can be applied using two methods. The opening method involves making an artificial hole in the ZP, while the thinning method involves using laser

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energy to thin the outer wall of the ZP. Some studies have reported comparisons between laser-assisted thinning (LAT) and control methods [3, 10] or between laser-assisted opening (LAO) and control methods [2, 6]. However, an insufficient number of studies have compared these two methods (i.e., thinning vs. opening) of LAH with the goal of identifying the optimal technique for clinical applications [8].

A recent retrospective study reported that no statistically significant differences between LAT and LAO. However, despite their results, they recommended the use of LAT because thinning was considered less traumatic and because they did not find that opening had any statistically significant advantages [11]. Therefore, we compared these two types of LAH is more effective in the rates of implantation and pregnancy outcomes in young maternal age (YMA; < 38 years) and old maternal age (OMA; ≥ 38 years) patients with RIF.

Materials and methods

Participants

A retrospective study was performed of 509 in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles (458 patients) from January 2013 to July 2017 at Agaon Fertility Clinic, Seoul, South Korea. The criterion of maternal age used the cutoff of 38 years, after which ovarian function is dramatically reduced, as previously described by Stoop et al. [12]. Our study included the following patient groups to minimize influences of the various infertility of patient on clinical outcomes: (i) had a least two episodes of implantation failure, and (ii) had an endometrial thickness ≥ 8 mm on the day of embryos transfer. Patients who underwent oocyte donation, oocyte activation, or genetic diagnosis were excluded from our data, as well as those who used surrogate mothers. Participants were divided into four groups according to maternal age and the types of LAH (YMA: LAT, $n = 119$ vs. LAO, $n = 179$ and OMA: LAT, $n = 72$ vs. LAO, $n = 139$) (Fig. 1). This study was approved by the Institutional Review Board (IRB reference number, Agaon IRB-17-003) of Agaon Fertility Clinic.

Semen collection, semen analysis, and sperm preparation

All men were identified before collecting semen samples. Semen samples were collected into a sterilized wide-mouthed plastic container by masturbation and analyzed after 30 min liquefaction on a slide warmer at 35 °C. The viscosity in the semen sample was estimated by gentle aspiration using a plastic disposable pipette, and then the semen was dropped by gravity and confirmed the length of the viscosity. The semen volume was measured directly by aspirating the sample from the specimen container into a pipette. Total sperm concentration, motility, and progressive motility were analyzed using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) [13] according to the 2010 World Health Organization (WHO) guidelines [14]. We immediately assessed sperm motility within 30 min after liquefaction in order to limit the damage influences of dehydration, pH, or a temperature change on sperm motility. The motility of each sperm was analyzed according to the following criteria: Progressive motility of sperm is moving actively, either linearly or in a large circle irrespective of sperm speed. Non-progressive motility of sperm is all other motility patterns without a progression (i.e., swimming in small circles, the flagella force hardly displacing the head, or when only the motion of the flagella was observed). Immotility of sperm is no movement. Sperm preparation was performed using the swim-up technique, as previously described by Younglai

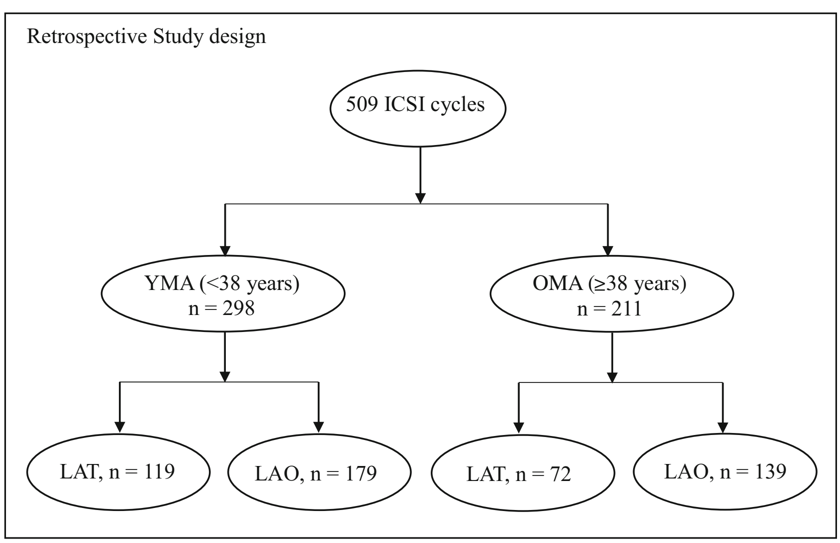

Fig. 1 Flow diagram of the retrospective study design. YMA, young maternal age; OMA, old maternal age; LAT, laser-assisted thinning; LAO, laser-assisted opening

et al. [15]. One milliliter of the raw semen was placed in 5 mL round bottom tube, and then 1 mL of sperm medium was overlaid on the raw semen. After direct swim-up for 20 min, the supernatant with motile sperm was carefully collected and transferred into a 15 mL conical tube. Concentrated the sperm by centrifugation at 1500 rpm for 5 min and then stored the tube at room temperature before ICSI.

Ovarian stimulation and IVF/ICSI procedures

All study participants underwent either a gonadotropin-releasing hormone (GnRH) agonist long protocol or a GnRH antagonist (Cetrotide, Merck Serono, Roma, Italy) protocol. Human chorionic gonadotropin (hCG; Ovidrel, Merck Serono) was administered when the dominant follicles reached 18 mm in diameter. Oocytes were collected 36 h after hCG administration under transvaginal ultrasound guidance with a 19-gauge needle (Dukwoo Medical, Hwaseong, Korea). Cumulus cell masses around the oocytes were removed using pull-and-cut denudation pipettes in a 5-well culture dish (MTG, Bruckberg, Germany) containing 27 IU/mL of hyaluronidase. All oocytes that were mature at 4–6 h after oocyte collection were inseminated according to the quality of the spermatozoa and oocytes and the patient's previous IVF history [16]. Fertilization was confirmed 17–18 h after insemination by the presence of two distinct pronuclei. Zygotes were cultured in 30-μL micro-drops with a 1-step medium (Life Global, Belgium) and overlaid with paraffin oil (Vitrolife, Gothenburg, Sweden) in an atmosphere of 6% CO_2, 5% O_2, and 95% humidity at 37 °C.

Embryo transfer and LAH

On day 3, either two or three embryos were transferred to the uterus. The choice of the number of embryos to be transferred was made following the criteria recommended by the Ministry of Health and Welfare of Korea. The available embryos were assessed in all patients according to the criteria of equal and regular blastomeres, a viable blastomere number, and fragmentation ratio, and embryo transfer was performed using a catheter (COOK Medical, Brisbane, Australia).

LAH was conducted using the ZILOS-tk Clinical Laser System (Hamilton Thorne Instruments Biosciences, Beverly, MA, USA), with a 1.46-μm diode-laser at 300 m W through thinning or opening. The laser system was integrated into the × 40 objective combination. The laser-objective was attached directly to the turret of a Nikon Eclipse Ti-U inverted microscope (Nikon Instruments Europe B.V., Badhoevedorp, Netherlands). The embryos underwent either LAT or LAO in 10- to 20-μL micro-drops consisting of G-MOPS plus medium (Vitrolife). The embryos were fixed in the micro-drops on the inverted microscope. The size of one laser shot was 5 μm in the ZP using the same total number of 3000-μs pulses. In LAT, the goal of the thinning method was to make the outer portion of the outer protective glycoprotein layer thinner by a certain amount. The laser thinning was performed by making 2–3 holes without reaching the inner membrane at a depth of 60–80% of the ZP thickness. In LAO, the laser opening was made from the outside to the inside of the ZP (Fig. 2). The same protocol was conducted, as described by Mantoudis et al. [8]. Hartshorn et al. (2005) found no increase in hsp70i in embryos conducted by the laser [35]. However, other studies have reported that if embryos are heated above 37 °C then heat shock protein (hsp) 70i transcription has risen which could potentially induce the risk of the embryo development [33, 34]. Therefore, the laser beam was shot towards the ZP above the perivitelline space between two blastomeres to minimize the risk of harming the embryo, under careful control. The embryos in the micro-drop were washed several times after LAH and then transferred to a 1-well dish (Falcon 353,653, USA) containing G-2 culture medium (Vitrolife). LAH was performed 2 to 3 h before embryo transfer. The embryos were cultured in an atmosphere of 6% CO_2, 5% O_2, and 95% humidity at 37 °C.

Clinical outcomes

Patients' endometria were prepared according to artificial cycle protocols with exogenous estrogens, as previously described by Lee et al. [17]. Progesterone in oil (50 mg/day) was administered daily until an endometrial thickness of 8 mm or more was reached. Biochemical pregnancy was regarded as a positive β-hCG level within 14 days after the ovum pick-up. Clinical pregnancy and successful implantation were indicated by the presence of as a gestational sac and fetal cardiac activity at 6–7 weeks of gestation after a positive β-hCG test. Abortion was defined as complete fetal loss after confirmation of a gestational sac using ultrasound at 12 weeks of gestation. Ongoing, singleton and twin pregnancies were defined as those that progressed at 12 weeks of gestation with fetal cardiac activity.

Statistical analysis

Outcome measures were compared between LAT and LAO within patient groups defined by maternal age. Statistical analysis was performed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). The statistical differences between groups were assessed with the independent-samples *t* test for parametric data. Categorical data were analyzed using the chi-square test. The values were presented as the mean ± standard deviation or number/total (%). P values < 0.05 were considered to indicate significant differences.

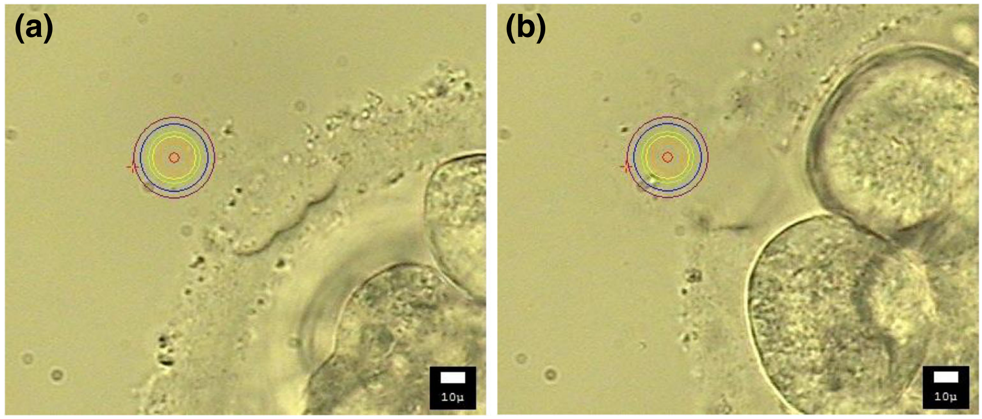

Fig. 2 Photographs of two types of laser-assisted hatching. **a** Laser-assisted thinning; **b** laser-assisted opening (× 400)

Results

We investigated a total of 509 cycles in this study. Patients were transferred 2 embryos in each cycle. As shown in Table 1, no significant differences were observed in terms of female age (33.6 ± 2.3 vs. 33.9 ± 2.3), male age (35.8 ± 3.2 vs. 35.5 ± 3.2), the number of previous IVF attempts (2.5 ± 0.7 vs. 2.6 ± 0.8), and the type of infertility diagnosis between LAT and LAO in the YMA group ($p > 0.05$), with the exception of mixed-factor infertility, which was more common in LAT than LAO in the YMA group (10.1% vs. 2.8%, $p = 0.008$). Nevertheless, the numbers of oocytes retrieved (8.8 ± 5.9 vs. 8.9 ± 5.2), oocytes matured (93.9% vs. 92.1%), and oocytes fertilized (75.6% vs. 74.2%) were similar between LAT and LAO in the YMA group ($p > 0.05$). Additionally, the mean female age (40.4 ± 2.2 vs. 40.6 ± 2.2), male age (41.1 ± 5.6 vs. 41.3 ± 3.5), the number of previous IVF attempts (2.6 ± 0.8 vs. 2.6 ± 0.8), and the type of infertility diagnosis were similar between LAT and LAO in the OMA group ($p > 0.05$). The numbers of oocytes retrieved (5.4 ± 3.6 vs. 6.0 ± 3.8), oocytes matured (91.0% vs. 91.3%), and oocytes fertilized (77.2% vs. 78.1%) were also similar between LAT and LAO in the OMA patients ($p > 0.05$).

As shown in Table 2, the rates of biochemical pregnancy (37.0% vs. 30.7%), clinical pregnancy (27.7% vs. 24.6%), ongoing pregnancy (22.7% vs. 21.8%), abortion (18.2% vs. 11.4%), implantation (17.2% vs. 16.5%), singleton pregnancy (17.6% vs. 16.2%), and twin pregnancy (5.0% vs. 5.6%) were not significantly different between LAT and LAO in the YMA group ($p > 0.05$). Additionally, the rates of the biochemical pregnancy (30.6% vs. 25.2%), clinical pregnancy (16.7% vs. 18.7%), ongoing pregnancy (8.3% vs. 15.1%), abortion

Table 1 Characteristics of patients who underwent LAT versus LAO according to maternal age with repeated implantation failure

	Maternal age < 38 year			Maternal age ≥ 38 year		
	LAT	LAO	*p* value	LAT	LAO	*p* value
No. of cycles	119	179		72	139	
Female age (year)	33.6 ± 2.3	33.9 ± 2.3	0.283	40.4 ± 2.2	40.6 ± 2.2	0.401
Male age (year)	35.8 ± 3.2	35.5 ± 3.2	0.570	41.1 ± 5.6	41.3 ± 3.5	0.728
No. of IVF attempts	2.5 ± 0.7	2.6 ± 0.8	0.297	2.6 ± 0.8	2.6 ± 0.8	0.600
Infertility diagnosis						
Female factor	57 (47.9)	89 (49.7)	0.687	48 (66.7)	96 (69.1)	0.451
Male factor	10 (8.4)	24 (13.4)	0.183	1 (1.4)	2 (1.4)	0.950
Mixed factor	12 (10.1)*	5 (2.8)	0.008	10 (13.9)	12 (8.6)	0.280
Unexplained factor	40 (33.6)	61 (34.1)	0.934	13 (18.1)	29 (20.9)	0.535
No. of oocytes retrieved	8.8 ± 5.9	8.9 ± 5.2	0.842	5.4 ± 3.6	6.0 ± 3.8	0.324
No. of oocytes matured	981/1045 (93.9)	1469/1595 (92.1)	0.084	356/391 (91.0)	758/830 (91.3)	0.873
No. of oocytes fertilized	742/981 (75.6)	1090/1469 (74.2)	0.422	275/356 (77.2)	592/758 (78.1)	0.749
No. of embryos transferred	2	2	NA	2	2	NA

Values are presented as mean ± standard deviation or number/total (%)

[*] Significant difference

LAT laser-assisted zona thinning; *LAO* laser-assisted zona opening; *IVF* in vitro fertilization; *NA* not applicable

Table 2 Pregnancy outcomes of LAT versus LAO according to maternal age with repeated implantation failure

	Maternal age < 38 year			Maternal age ≥ 38 year		
	LAT	LAO	*p* value	LAT	LAO	*p* value
Biochemical pregnancy	44 (37.0)	55 (30.7)	0.262	22 (30.6)	35 (25.2)	0.404
Clinical pregnancy	33 (27.7)	44 (24.6)	0.543	12 (16.7)	26 (18.7)	0.715
Ongoing pregnancy	27 (22.7)	39 (21.8)	0.854	6 (8.3)	21 (15.1)	0.163
Abortion	6 (18.2)	5 (11.4)	0.397	6 (50.0)	5 (19.2)	0.052
Implantation*	41 (17.2)	59 (16.5)	0.811	16 (11.1)	31 (11.2)	0.990
Singleton pregnancy	21 (17.6)	29 (16.2)	0.744	6 (8.3)	18 (12.9)	0.317
Twin pregnancy	6 (5.0)	10 (5.6)	0.498	0 (0.0)	3 (2.2)	0.553

Values are presented as number or number/total (%)

*Implantation is presented as number of gestational sac per number of embryos transferred (%)

LAT laser-assisted zona thinning; *LAO* laser-assisted zona opening

(50.0% vs. 19.2%), implantation (11.1% vs. 11.2%), singleton pregnancy (8.3% vs. 12.9%), and twin pregnancy (0.0% vs 2.2%) were also similar between LAT and LAO in the OMA group ($p > 0.05$).

Discussion

In this study, we investigated whether there were any differences in the clinical outcomes between LAT and LAO in patients with RIF according to maternal age. No statistically significant differences in clinical outcomes were found between the two methods in either the YMA or the OMA group.

The premise of this study was based on the findings of previous studies that AH could improve the rates of implantation and pregnancy in OMA patients and patients with RIF [2, 6, 26]. Blastocysts must hatch from the ZP before being implanted in the endometrium. The hypothesis underlying the AH is that modifying the ZP might improve the hatching and implantation rates of embryos that do not have the ability to escape from the ZP [1, 18]. Since then, many studies have applied AH to treat women whose embryos have undergone chemical or physical ZP changes. However, despite the efforts of many researchers, discordances persist in the results of AH [19–21], and some studies have reported that some patients observed no benefits from the use of AH [3, 4, 22]. These discrepancies are thought to be due to differences in AH methods and techniques, experimental design, freezing methods, and patient characteristics [23].

AH can be performed using mechanical, chemical, or laser energy. The mechanical method uses a micromanipulator with an injection and a holding pipette. The embryos are held in position by the holding pipette, the injection pipette is passed through the ZP, and then the embryo is gently rubbed until the ZP is opened [24]. The processing time of the mechanical method is fast, but the size of the hole in the ZP is not consistent. The chemical method is performed by preloading an acid Tyrode's solution–filled microneedle at the 3 o'clock position and injecting it in either the empty perivitelline space or extracellular fragments [25]. The chemical method requires the skill of an experienced researcher because of the need to minimize exposure of the embryos to the toxic acid solution. Lastly, LAH can be applied through an opening or thinning [6, 9]. Compared with the chemical and mechanical procedures, LAH has the advantage of enabling the easy removal of the ZP by approaching the embryos in a non-contact mode. The laser light delivery system has the advantage of being able to access the target accurately with minimal absorption by the embryo. Furthermore, the laser target is applied to thin or open the ZP without mechanical or mutagenic side effects [7, 8]. A review reported that LAH is currently one of the safest and most effective methods of AH [9].

Previous studies have compared LAT or LAO with other methods [3, 10], rather than comparing the two LAH methods [2, 6]. Therefore, an insufficient number of studies have directly compared these two laser-based methods [8]. A recent retrospective study reported no statistically significant differences between the two methods [11]. Their results are consistent with ours. However, despite their results, they recommended the use of LAT, because LAT involves a smaller hole in the ZP, which is thought to be less likely to lead to embryo damage than the larger hole created by zona drilling/opening [24]. Additionally, embryos opened using acidic Tyrode's solution and partial zona dissection may undergo a first division, potentially increasing the incidence of monozygotic twin pregnancies after embryo transfer [27, 28]. In contrast, LAT is considered less traumatic and may offer advantages over mechanical and chemical AH methods of making holes [29]. However, according to previous studies, LAT alone is not sufficient to induce complete blastocyst hatching and to promote implantation, suggesting that the inner layer of the ZP should be completely removed [8, 30]. In a recent mouse study using a time-lapse system, the frequency of complete hatching was 33.9% in the zona-intact control group, 94.4% after LAO, and 39.3% after LAT ($p < 0.0001$). In total, 60.7% of the zona-thinned mouse embryos did not complete the hatching process and remained trapped in the zona-thinned area, and embryos also hatched in areas where the ZP was not thinned. As a result, the researchers suggested that LAT should not be considered as a method for AH [31]. Furthermore, a recent prospective randomized study reported that adequate LAO improved the subsequent hatching rate in studies using a time-lapse system [32]. According to the results of those studies, LAT may increase the incidence of monozygotic twin pregnancies to a greater extent than LAO.

We did not find any evidence that LAO posed a high risk of damage to the embryo, and the laser targeting method used in our study also a minimized the risk of damage to the embryo. We think that removing the ZP with a uniform thickness using LAT, regardless of size, may require a long period of work time for less experienced embryologists. It has been previously reported that LAO can minimize the external exposure of the embryo due to the short period of work time [23]. In our data, although there were no statistically significant differences between LAO and LAT, the OMA patients who underwent LAO tended to have a lower abortion rate.

This study has some limitations. The number of LAT cycles was smaller than the number of LAO cycles. However, the total number of embryos transferred (1018 embryos) in this study was larger than a previous retrospective study (432 embryos) [11]. Secondly, we did not compare LAO with various amounts of LAT. The ZP area of LAH was approximately the same in the LAT and LAO groups. Further research is needed to compare the effects of LAO and various amounts of LAT on clinical outcomes, as results regarding the amount of LAT in the ZP of human embryos remain inconclusive [23]. To the best of our knowledge, this is the first published study to focus on a comparison of two types of LAH (i.e., LAT and LAO) according to the maternal age (i.e., < 38 years and ≥ 38 years) of patients with at least two episodes of implantation failure.

In conclusion, LAO and LAT had similar clinical outcomes regardless of maternal age in patients with RIF. Therefore, we did not find any evidence indicating that LAT is superior to LAO. In addition, the OMA patients who underwent LAO tended to show a lower abortion rate. Further study is necessary to confirm these results in a larger population.

Acknowledgments The authors express special thanks to the staff of the Agaon Fertility Clinic.

Funding This study was self-funded.

Compliance with ethical standards

This study was approved by the Institutional Review Board (IRB reference number, Agaon IRB-17-003) of Agaon Fertility Clinic.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Cohen J, Malter H, Fehilly C, Wright G, Elsner C, Kort H, Massey J (1988) Implantation of embryos after partial opening of oocyte zona pellucida to facilitate sperm penetration. Lancet 332(8603):162
2. Hsieh Y, Huang C, Cheng T, Chang C, Tsai H, Lee M (2002) Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. Fertil Steril 78(1):179–182
3. Frydman N, Madoux S, Hesters L, Duvernoy C, Feyereisen E, Le Du A, Tachdjian G, Frydman R, Fanchin R (2006) A randomized double-blind controlled study on the efficacy of laser zona pellucida thinning on live birth rates in cases of advanced female age. Hum Reprod 21(8):2131–2135
4. Edirisinghe WR, Ahnonkitpanit V, Promviengchai S, Suwajanakorn S, Pruksananonda K, Chinpilas V, Virutamasen P (1999) A study failing to determine significant benefits from assisted hatching: patients selected for advanced age, zonal thickness of embryos, and previous failed attempts. J Assist Reprod Genet 16(6):294–301
5. Petersen CG, Mauri A, Baruffi R, Oliveira J, Massaro F, Elder K, Franco J (2005) Implantation failures: success of assisted hatching with quarter-laser zona thinning. Reprod BioMed Online 10(2):224–229
6. Valojerdi MR, Eftekhari-Yazdi P, Karimian L, Ashtiani SK (2008) Effect of laser zona pellucida opening on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen–thawed embryos. Fertil Steril 90(1):84–91
7. Germond M, Nocera D, Senn A, Rink K, Delacrétaz G, Fakan S (1995) Microdissection of mouse and human zona pellucida using a 1.48μm diode laser beam: efficacy and safety of the procedure. Fertil Steril 64(3):604–611
8. Mantoudis E, Podsiadly BT, Gorgy A, Venkat G, Craft IL (2001) A comparison between quarter, partial and total laser assisted hatching in selected infertility patients. Hum Reprod 16(10):2182–2186
9. Hammadeh ME, Fischer-Hammadeh C, Ali KR (2011) Assisted hatching in assisted reproduction: a state of the art. J Assist Reprod Genet 28(2):119–128
10. Kutlu P, Atvar O, Vanlioglu OF (2010) Laser assisted zona thinning technique has no beneficial effect on the ART outcomes of two different maternal age groups. J Assist Reprod Genet 27(8):457–461
11. Padula F, Capriglione S, Iaconianni P, Gatti S, Lippa A, Minutolo SE, Giannarelli D, Giorlandino C (2017) Laser-assisted hatching of human embryos: may two alternative approaches (thinning versus drilling) impact on implant rate. Lasers Med Sci 32(7):1663–1666
12. Stoop D, Ermini B, Polyzos NP, Haentjens P, De Vos M, Verheyen G, Devroey P (2012) Reproductive potential of a metaphase II oocyte retrieved after ovarian stimulation: an analysis of 23 354 ICSI cycles. Hum Reprod 27(7):2030–2035
13. Cardona-Maya W, Berdugo J, Cadavid A (2008) Comparing the sperm concentration determined by the Makler and the Neubauer chambers. Actas Urol Esp 32(4):443–445
14. World Health Organ (ed) (2010) WHO laboratory manual for the examination and processing of human semen, 5th edn. World Health Organization, Geneva
15. Younglai EV, Holt D, Brown P, Jurisicova A, Casper RF (2001) Sperm swim-up techniques and DNA fragmentation. Hum Reprod 16(9):1950–1953
16. Lee JW, Cha JH, Shin SH, Kim YJ, Lee SK, Cha HJ, Kim JH, Ahn JH, Kim HY, Pak KA, Yoon JS, Park SY, Park CK (2016) Long cut straw provides stable the rates of survival, pregnancy and live birth for vitrification of human blasotcysts. Dev Reprod 20(3):219–225

17. Lee JW, Cha JH, Shin SH, Kim YJ, Lee SK, Park CK, Pak KA, Yoon JS, Park SY (2017) Efficacy of embryo transfer on day 2 versus day 3 according to maternal age in patients with normal ovarian response. Clin Exp Reprod Med 44(3):141–145
18. Cohen J, Malter H, Wright G, Kort H, Massey J, Mitchell D (1989) Partial zona dissection of human oocytes when failure of zona pellucida penetration is anticipated. Hum Reprod 4(4):435–442
19. Antinori S, Selman H, Caffa B, Panci C, Dani G, Versaci C (1996) Zona opening of human embryos using a non-contact UV laser for assisted hatching in patients with poor prognosis of pregnancy. Hum Reprod 11(11):2488–2492
20. Magli MC, Gianaroli L, Ferraretti AP, Fortini D, Aicardi G, Montanaro N (1998) Rescue of implantation potential in embryos with poor prognosis by assisted zona hatching. Hum Reprod 13(5): 1331–1335
21. Nakayama T, Fujiwara H, Yamada S, Tastumi K, Honda T, Fujii S (1999) Clinical application of a new assisted hatching method using a piezo-micromanipulator for morphologically low-quality embryos in poor-prognosis infertile patients. Fertil Steril 71(6):1014–1018
22. Bider D, Livshits A, Yonish M, Yemini Z, Mashiach S, Dor J (1997) Assisted hatching by zona drilling of human embryos in women of advanced age. Hum Reprod 12(2):317–320
23. Wang E, Wang A, Wang B, Li B (2016) Outcomes of vitrified-warmed cleavage-stage embryo hatching after in vitro laser-assisted zona pellucida thinning in patients. Biomed Rep 5(3): 376–382
24. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, Wiemer K (1990) Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod 5(1):7–13
25. Cohen J (1991) Assisted hatching of human embryos. J In Vitro Fert Embryo Transf 8(4):179–190
26. Martins WP, Rocha IA, Ferriani RA, Nastri CO (2011) Assisted hatching of human embryos: a systematic review and meta-analysis of randomized controlled trials. Hum Reprod Update 17(4):438–453
27. Alikani M, Noyes N, Cohen J, Rosenwaks Z (1994) Monozygotic twinning in the human is associated with the zona pellucida architecture. Hum Reprod 9(7):1318–1321
28. Luke B, Brown MB, Wantman E, Stern JE (2014) Factors associated with monozygosity in assisted reproductive technology pregnancies and the risk of recurrence using linked cycles. Fertil Steril 101(3):683–689
29. Blake D, Forsberg A, Johansson B, Wikland M (2001) Laser zona pellucida thinning—an alternative approach to assisted hatching. Hum Reprod 16(9):1959–1964
30. Tucker MJ, Luecke NM, Wiker SR, Wright G (1993) Chemical removal of the outside of the zona pellucida of day 3 human embryos has no impact on implantation rate. J Assist Reprod Genet 10(3):187–191
31. Schimmel T, Cohen J, Saunders H, Alikani M (2014) Laser-assisted zona pellucida thinning does not facilitate hatching and may disrupt the in vitro hatching process: a morphokinetic study in the mouse. Hum Reprod 29(12):2670–2679
32. Watanabe H, Suzuki R, Kobayashi M, Hasegawa H, Tsukamoto K, Saitou S, Kobayashi J (2015) To what degree should the zona pellucida be cut open in assisted hatching for best clinical results? Fertil Steril 104(3):e185
33. Hartshorn C, Anshelevich A, Wangh LJ (2005) Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. BMC Biotechnol 5:2
34. Al-Katanani YM, Hansen PJ (2002) Induced thermotolerance in bovine two-cell embryos and the role of heat shock protein 70 in embryonic development. Mol Reprod Dev 62(2):174–180
35. Hartshorn C, Anshelevich A, Wangh LJ (2005) Laser zona drilling does not induce hsp70i transcription in blastomeres of eight-cell mouse embryos. Fertil Steril 84(5):1547–1550